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Antacid Increases Survival of *Vibrio vulnificus* and *Vibrio vulnificus* Phage in a Gastrointestinal Model†

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Viable counts of three strains of *Vibrio vulnificus* and its phage were determined during exposure to a mechanical gastrointestinal model with or without antacid for 9 h at 37°C. *V. vulnificus* was eliminated (>4-log reduction) within 30 min in the gastric compartment (pH decline from 5.0 to 3.5). Viable *V. vulnificus* cells delivered from the gastric compartment during the first 30 min of exposure reached 10^6 to 10^8 CFU/ml in the intestinal compartment after 9 h (pH 7.0). Phages were eliminated within 45 min in the gastric compartment (pH decline from 5.1 to 2.5). Less than a 2-log reduction of phage was observed in the intestinal compartment after 9 h (pH 7.0). When the gastric compartment contained antacid *V. vulnificus* counts decreased slightly (<2 log) during 2 h of exposure (pH decline from 7.7 to 6.0), while counts in the intestinal compartment (pH 7.5) reached 10^7 to 10^9 CFU/ml. Phage numbers decreased 1 log after 2 h in the gastric compartment (pH decline from 7.7 to 5.7) containing antacid and decreased 1 log in the intestinal compartment (pH 7.6) after 9 h. Presence of antacid in the gastric compartment of the model greatly increased the ability of both *V. vulnificus* and its phage to survive simulated gastrointestinal transit and may be a factor involved with oyster-associated illness.

Vibrio vulnificus is a virulent pathogen (3, 42). It is most commonly found in estuarine and marine waters of the U.S. Gulf Coast and other temperate regions (7, 33). Of all food-borne infectious diseases in the United States, *V. vulnificus* has the highest (0.39) case fatality rate (31). Disease can follow the ingestion of raw Gulf Coast oysters and may result in primary septicemia or gastroenteritis in individuals who have underlying chronic disease, including liver disease (37). Wound infections are associated with exposure of wounds to seawater or shellfish.

Most-probable numbers of *V. vulnificus* organisms in Gulf Coast oysters range from 10^2 to 10^4/g from April through October, while most-probable numbers of <10/g occur during the winter (33). Bacteriophages lytic to *Vibrio* species also are prevalent throughout the Gulf of Mexico (26); they are found in estuarine water samples (35) and in a variety of oyster tissues (8). *V. vulnificus* phage numbers range from 10^4 to 10^5 PFU/g in Gulf Coast oysters throughout the year (9).

Microorganisms causing human gastroenteritis must survive the gastric barrier, resist bile in the small intestine, and colonize the intestinal lumen. The gastric barrier is lethal to most ingested bacteria (10, 19, 20), including *V. vulnificus* (27, 28). *Vibrio cholerae* is an acid-sensitive pathogen compared to other enteric pathogens such as *Salmonella enterica* serovar Typhi, *S. enterica* serovar Typhimurium, *Shigella flexneri*, and *Escherichia coli* O157:H7 (44). *V. cholerae* does not survive in either pH 4.0 or 5.0 broth after 2 h of exposure. Likewise, *V. vulnificus* also is acid sensitive in pH 4.0 broth (27). Simulated gastric fluid (SGF) is more inhibitory to *V. vulnificus* than acidified broth (28).

Antacids neutralize stomach acidity and are widely used for relief of gastric ulcers, duodenal ulcers, heartburn, and acid indigestion (13, 14, 16, 29, 38, 45). Antacids neutralize acid in the human stomach for a short duration, while acid blockers reduce acid secretion for a prolonged duration (14). Reduction of gastric acidity results in a substantial increase in survival rates of common food-borne pathogens (36). Reduced gastric acidity by medications may favor increased survival and subsequent growth of *V. vulnificus*, which could increase the risk of infection. In human volunteers, the dose of *V. cholerae* required to induce diarrhea was lowered from 10^8 to 10^4 organisms by neutralizing stomach acidity with the antacid $NaHCO_3$ (4). Despite these reports, an epidemiological study of *V. vulnificus* reported that use of antacids or cimetidine, an acid blocker, were not significant risk factors for primary sepsis (42).

The ability to tolerate low gastric pH and to resist intestinal bile is necessary for *V. vulnificus* to cause food-borne infections in humans. Previous reports also discussed the effectiveness of phage therapy applications to treat enteropathogenic *E. coli* diarrhea in calves (39) and *S. enterica* serovar Typhimurium in chickens (2). Thus, phages may be an important factor affecting *Vibrio* populations in estuarine environments and in the gastrointestinal tract of humans. The objectives of the present study were to assess survival of *V. vulnificus* and its phage in oysters during in vitro transit in a gastrointestinal (GI) model (12, 30, 32, 34) and to determine the influence of antacid on survival of *V. vulnificus* and its phage in the GI model.

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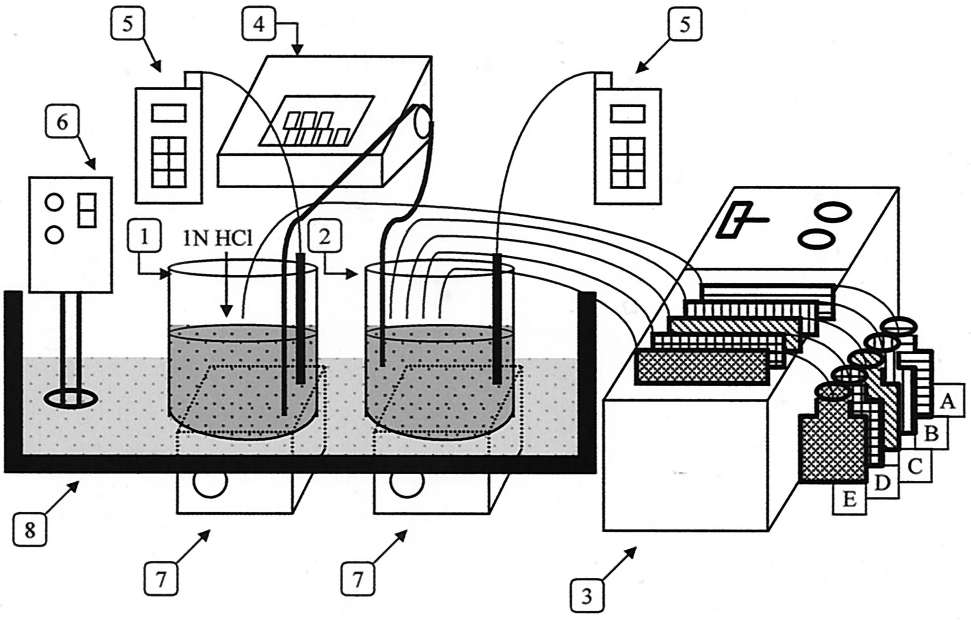

FIG. 1. Diagram of a simulated dynamic-GI model. Components: 1, GC; 2, IC; 3, cassette pump; 4, peristaltic pump; 5, pH meters; 6, circulator-heater; 7, stirrers; 8, water bath. Fluids: A, SGF; B, SIF; C, $NaHCO_3$; D, 2% bile; E, 4% bile.

MATERIALS AND METHODS

Bacterial cultures and phages. *V. vulnificus* strains A-9 (environmental isolate), MO6-24 (clinical isolate), and 304 (oyster isolate) were maintained at room temperature on T_1N_1 agar slants containing 10 g of tryptone (Difco Laboratories, Detroit, Mich.), 10 g of NaCl, 20 g of Bacto Agar (Difco), and 1.0 liter of distilled water. Cultures were transferred biweekly to maintain viability. Strains were streaked on tryptic soy agar (Difco) plates containing 2% NaCl (TSA-2) and incubated overnight at 37°C. Several isolated colonies of each strain were picked using sterile loops and suspended in phosphate-buffered saline (43). The bacterial suspension was adjusted to a turbidity of 3.5 to 3.8 nephelometric turbidity units (Hach Turbidimeter; Hach Co., Loveland, Colo.) to achieve a concentration of 10^7 CFU/ml. *V. vulnificus* phage strains 154A-9, 153A-7, and 110A-7 (all podophages of Bradley group C-3 morphology from Gulf Coast oysters) were maintained and prepared for inoculation as described previously (27, 28).

Preparation of GI model. The following equipment was used for the GI model (Fig. 1): a 250-ml Pyrex beaker gastric compartment (GC) with a pH meter probe (Orion 290A; Orion Research Inc., Boston, Mass.), a 250-ml Pyrex beaker small intestine compartment (IC) with a pH meter probe (Fisher Accumet 1001; Fisher Scientific, Pittsburgh, Pa.), a peristaltic pump (Wheaton Unispense; Wheaton Instruments, Millville, N.J.), a cassette pump (Manostat, New York, N.Y.), two magnetic stirrers (Fisher), and a 37°C Isotemp Immersion Circulator model 730 water bath (Fisher). The two beakers with magnetic stirring bars were placed in the water bath and were connected by the peristaltic pump for gastric emptying from the GC to the IC. Throughout the experiment, magnetic stirrers mixed samples in each compartment at low speed. SGF, simulated intestinal fluid (SIF), and bile solution were introduced into the GC or the IC by the cassette pump. The pH of the GC or the IC was monitored and adjusted by adding IN HCl manually into the GC and 0.1 or 0.3 M $NaHCO_3$ via the cassette pump into the IC.

Shucked raw oysters (*Crassostrea virginica*) were obtained from a local grocery store and kept frozen at −20°C until used. Autoclaved (121°C for 15 min) oysters (approximately 70 g) were mixed (1:1, wt/wt) with sterile electrolyte solution (32) containing 6.2 g of NaCl, 2.2 g of KCl, 0.22 g of $CaCl_2$, 1.2 g of $NaHCO_3$, and 1.0 liter of distilled water in a Waring blender (Dynamics Corporation Division, New Hartford, Conn.) for 2 min at high speed to simulate mouth chewing with saliva. This oyster homogenate was passed through a sterile filtering funnel to remove large particles to facilitate pumping from the GC to the IC. One hundred milliliters of oyster homogenate and 1 ml of bacterial or phage suspension were added into the GC to achieve a final concentration of approximately 10^5 CFU/ml or PFU/ml, respectively. All pumps were started simultaneously immediately after inoculation.

SGF contained 0.1 g of pepsin (Sigma Chemical Co., St. Louis, Mo.), 3.5 g of mucin (Sigma), 8.5 g of NaCl, and 1.0 liter of distilled water adjusted to pH 2.0 with 1 N HCl (23). SGF was pumped into the GC via the cassette pump at a flow rate of 0.33 ml/min. A predetermined constant volume (approximately 1.2 ml) of the chyme (mixture of food with SGF) was delivered from the GC to the IC every minute for 2 h via the peristaltic pump. The emptied chyme was collected continuously in the IC. Gastric in vivo pH values (5, 32) were obtained by manually adjusting the pH of the GC every 10 min using sterile 1 N HCl. GC pH values were measured every 10 min for 2 h. The half-emptying time of this GI model was 61 min, which was similar to normal gastric half-emptying time based on data from Ghoos et al. (18). SIF contained 0.1 g of trypsin (Sigma), 3.5 g of pancreatin (Sigma), and 1.0 liter of distilled water (23) and was pumped into the IC at a flow rate of 0.33 ml/min. Two different concentrations of $NaHCO_3$ were used to maintain a constant pH value in the IC (21). For the first 50 to 60 min, 0.1 M $NaHCO_3$ was pumped into the IC followed by 0.3 M $NaHCO_3$ for the rest of the experiment, both at a flow rate of 0.33 ml/min. IC pH values were measured for 3 of the 9 h of exposure because pH remained unchanged after 3 h (results not shown). Seven milliliters of 4% bile solution (ox gall; Sigma) was added into IC before the experiment began. A 2 or 4% bile solution also was pumped into the IC at a flow rate of 0.5 ml/min (28, 29, 30). A 4% bile solution was pumped during the first 30 min, and then 2% bile solution was pumped until the end of the experiment. Gastrointestinal fluids were pumped into both GC and IC until the contents of GC were emptied.

Influence of antacid in GI model. Aluminum hydroxide hydrate (692 mg) (32 to 35% water of hydration) (Sigma) and 400 mg of magnesium hydroxide (Fisher) were used as the antacid active ingredients found in 10 ml (2 teaspoonfuls) of Maalox (Ciba Self-Medication, Inc., Woodbridge, N.I.). They were added into the GC when bacterium or phage inocula and oyster homogenates were introduced. Gastric pH for antacid treatment was controlled by addition of 1 N HCl to GC to approximate normal gastric production. Sterile electrolyte solution was added in the IC to control intestinal pH for antacid treatment.

Microbiological analysis. One-milliliter samples were taken from the GC at 0, 10, 20, and 30 min for *V. vulnificus* or at 0, 15, 30, and 45 min for *V. vulnificus* phage. In GC containing antacid, samples were taken every 30 min for 2 h for both bacterium and phage. Samples were taken from IC every 1.5 h for 9 h for *V. vulnificus* and its phage. Samples for *V. vulnificus* enumeration were serially diluted in tryptic soy broth (Difco) containing 2% NaCl, and 0.1-ml aliquots were plated onto TSA-2. Samples for phage enumeration were serially diluted in sterile seawater and plated onto CPM agar containing Casamino Acids (5 g/liter; Difco), peptone (5 g/liter; Difco), 1.5% Bacto Agar (Difco), and 1 liter of seawater (Sigma) by using the soft-agar overlay technique containing *V. vulnifi-*

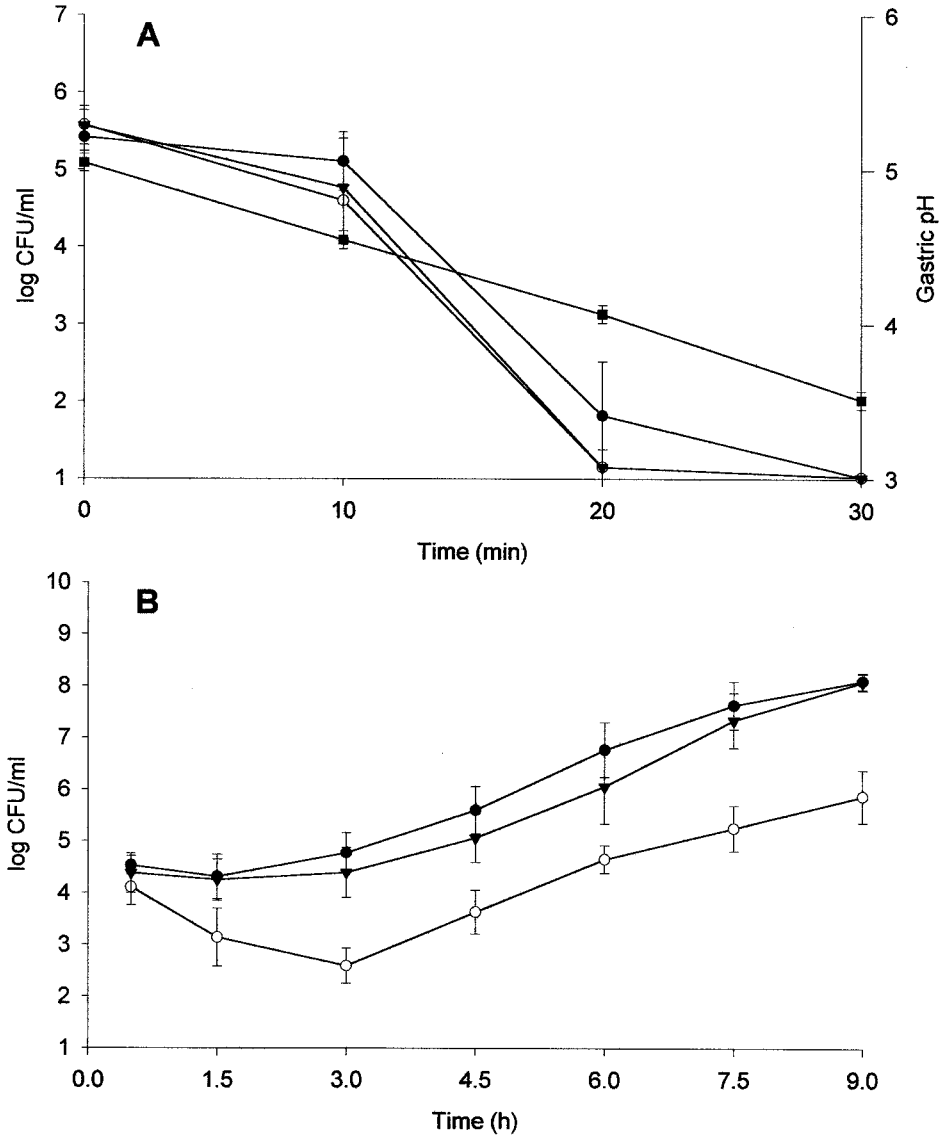

FIG. 2. Survival of *V. vulnificus* in the GC (A) and the IC (B) of the model at 37°C (●, *V. vulnificus* A-9; ○, *V. vulnificus* MO6-24; ▼, *V. vulnificus* 304; ■, pH). Error bars, standard deviations.

cus strain MO6-24 as host the culture (27, 28). All plates were incubated anaerobically (BBL Gas Pak Plus; Becton Dickinson, Cockeysville, Md.) at 37°C for 18 h. Viable cell or phage counts were divided by the dilution factors for GC or IC to take into account the volumes of secretions and emptied or delivered volumes at each sampling time interval. Dilution factors (all amounts in milliliters) were calculated as follows: dilution factor for GC = remaining GC contents/(remaining GC contents + HCl + SGF); dilution factor for IC = cumulative IC contents/(cumulative IC contents + delivered contents + bile + SIF + $NaHCO_3$). Microbial count data obtained from duplicate samples per analysis time from three replicate experiments were analyzed by ANOVA and means were separated by least-significant difference (SAS user's guide, 5th ed., SAS Institute Inc., Cary, N.C.)

RESULTS

Survival of *V. vulnificus*. Within 20 min, a 4-log reduction in *V. vulnificus* counts was observed in the normal GC at 37°C. No viable *V. vulnificus* cells (counts reduced below the limit of detection of 10 CFU/ml) were recovered after 30 min in the normal GC (Fig. 2A). No difference ($P > 0.05$) was detected among strains in the normal GC. Delivered cells were 4 to 4.5 $\log_{10}$ CFU/ml in the IC from the first 30 min of gastric emptying (Fig. 2B). Cell counts of strains A-9 and 304 increased continuously after 1.5 h and reached 8 $\log_{10}$ CFU/ml after 9 h at 37°C in the IC. In contrast, counts of *V. vulnificus* MO6-24 in the IC dropped 1.5 log after 3 h and then reached 6 $\log_{10}$ CFU/ml after 9 h (Fig. 2B). Strains A-9 and 304 in the IC grew faster ($P < 0.05$) than strain MO6-24. Gastric pH declined from 5.0 to 1.7 during 2 h of gastric emptying and intestinal pH ranged from 7.3 to 6.8 for 3 h, with no significant difference ($P > 0.05$) observed among trials using the three bacterial strains.

When antacid was added to the GC, numbers of strain A-9 and 304 organisms remained unchanged during 2 h of gastric exposure at 37°C, while a 2-log reduction was observed with

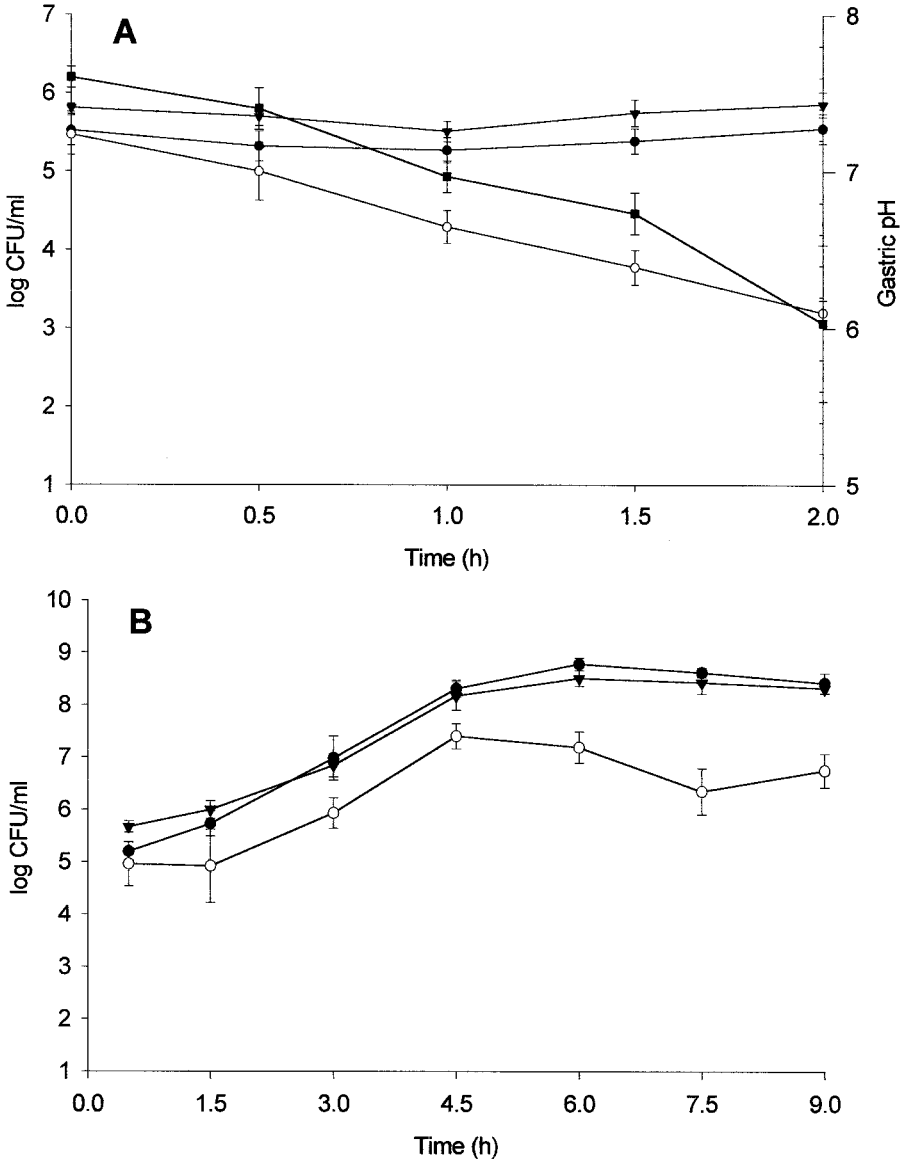

FIG. 3. Survival of *V. vulnificus* in the GC (A) and the IC (B) of the model containing antacid at 37°C (●, *V. vulnificus* A-9; ○, *V. vulnificus* MO6-24; ▼, *V. vulnificus* 304; ■, pH). Error bars, standard deviations.

strain MO6-24 (Fig. 3A). Cells delivered from the GC to the IC during 30 min of gastric emptying were 5 $\log_{10}$ CFU/ml in the IC, which was identical to the initial GC inoculum level. After 30 min in the IC, strains A-9 and 304 showed exponential growth and reached 8.5 to 9 $\log_{10}$ CFU/ml within 6 h, with no further growth up to 9 h (Fig. 3B). Numbers of strain MO6-24 organisms increased after 1.5 h and reached 7.5 $\log_{10}$ CFU/ml within 4.5 h, followed by a 0.5-log decrease up to 9 h (Fig. 3B). Strains A-9 and 304 showed better survival ($P < 0.05$) in the GC and better growth ($P < 0.05$) in the IC than did strain MO6-24. Gastric pH declined from 7.7 to 6.0 (Fig. 3A), while the IC pH was maintained at 7.5 (results not shown).

Survival of *V. vulnificus* phage. Phage counts decreased only 1 log during the first 30 min but were not recovered from the GC after 45 min at 37°C after gastric pH declined below 3.5 (Fig. 4A). Phage numbers delivered from the GC to the IC during the first 30 min of gastric emptying were 5 $\log_{10}$ PFU/ml, which was similar to the initial GC inoculum level (Fig. 4). Less than a 1.5-log phage count reduction was observed in the IC after 9 h at 37°C (Fig. 4B). For the entire GI model, phage numbers decreased less than 2 logs from the initial number. Phage counts were not significantly different ($P > 0.05$) among strains in both the GC and the IC. In trials with phages, gastric pH declined from 5.1 to 1.7 (Fig. 4A) and intestinal pH was maintained between 7.4 to 6.7 (results not shown), with no differences ($P > 0.05$) among strains tested.

With antacid, phage numbers decreased less than 1 log during the entire transit through the gastrointestinal model (Fig. 5). Phage counts were not significantly different ($P > 0.05$) among strains in both the GC and the IC containing antacid. Gastric pH declined from 7.7 to 5.7 (Fig. 5A), while the IC pH was maintained at 7.6 (results not shown).

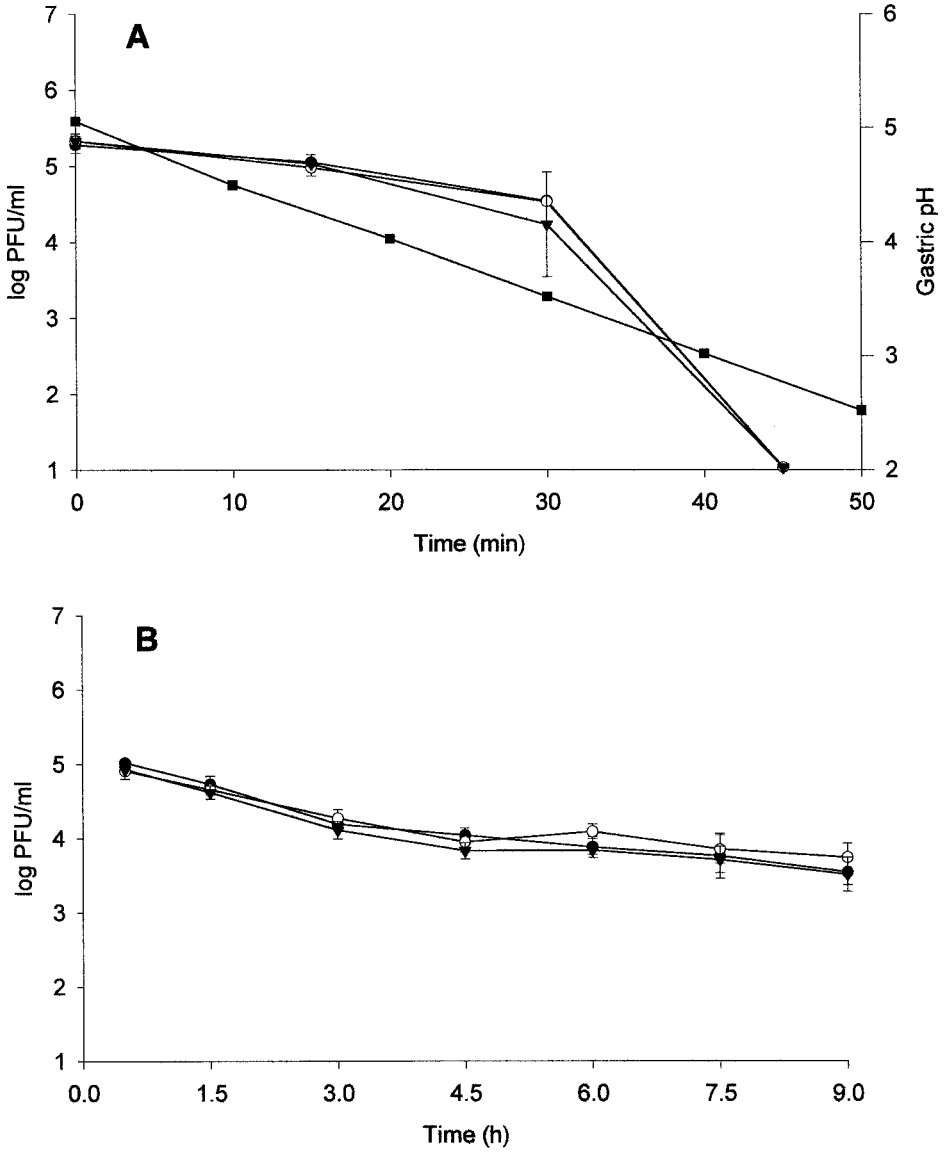

FIG. 4. Survival of *V. vulnificus* phage in the GC (A) and the IC (B) of the model at 37°C (●, *V. vulnificus* phage 154A-9; ○, *V. vulnificus* phage 153A-7; ▼, *V. vulnificus* phage 110A-7; ■, pH). Error bars, standard deviations.

DISCUSSION

Behavior of *V. vulnificus*. Several studies have reported that the bactericidal effect of gastric juice is reduced by the presence of food (5, 10, 36). Food particles protect bacteria in the stomach by enrobing cells and by buffering acid. In the present study with oysters, a 3-log reduction in *V. vulnificus* counts was observed between 10 and 20 min when pH of GC ranged between 4.5 and 4.1 (Fig. 2A), which was in agreement with previous observations with SGF without oysters (28). Thus, autoclaved oyster homogenate provided no apparent protection to the gastric barrier. Others have reported that *V. vulnificus* was more resistant to heat treatment in proteinaceous materials such as intermittently sterilized oyster and fish homogenates than in buffer (1).

V. vulnificus was not detected in the GC at 30 min when the pH value was below 3.5. A rapid gastric emptying rate may be an important factor for survival of *V. vulnificus* and risk of infection in the GI tract. The pylorus, the circular muscle of the end of the stomach, normally remains almost, but not completely, closed for partial discontinuity between the stomach and duodenum. The closing force is weak enough that water and other fluids empty from the stomach with ease (22). Davenport (6) reported that the rate of emptying for liquid meals is greatest when the volume is greatest so that emptying is fastest at the beginning of digestion of a meal. Consequently, the greatest bulk of gastric contents is delivered to the duodenum before much gastric digestion or acidification has occurred. The volume of liquid meals emptied into the duodenum is greatest during the first 10 to 20 min, with peak emptying rates occurring during the first hour after ingestion (30). Because oysters are semisolid and are frequently consumed as an appetizer, they would be expected to empty from

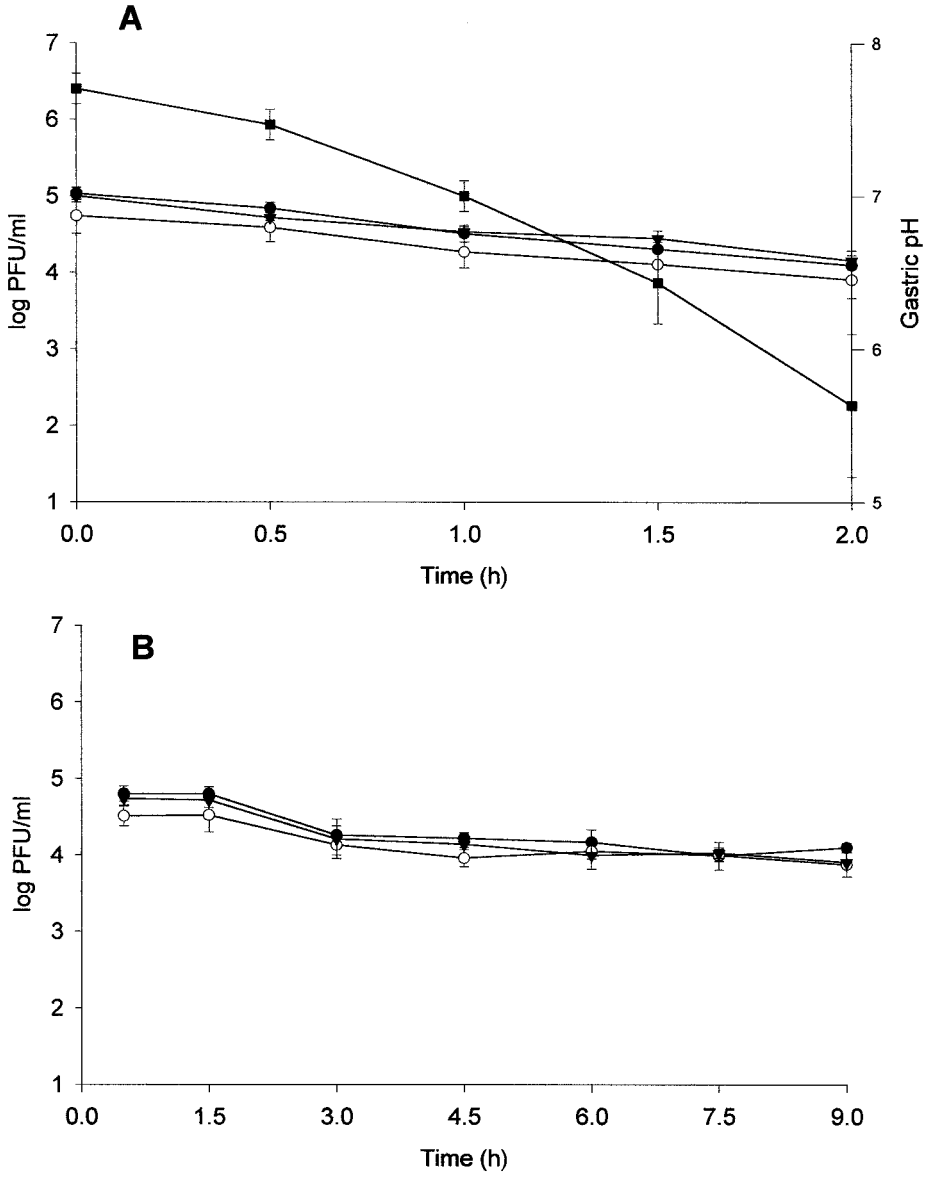

FIG. 5. Survival of *V. vulnificus* phage in the GC (A) and the IC (B) of the model containing antacid at 37°C (●, *V. vulnificus* phage 154A-9; O, *V. vulnificus* phage 153A-7; ▼, *V. vulnificus* phage 110A-7; ■, pH). Error bars, standard deviations.

the stomach easily and rapidly. In the present study, more than 80% of the inoculated *V. vulnificus* reached the IC as culturable cells during the first 30 min of gastric emptying when the gastric pH ranged from 5.0 to 3.5 (Fig. 2). While *V. vulnificus* reductions in the GC could be partially attributable to gastric emptying, acid inactivation likely accounted for at least a 4-log reduction (27, 28).

Present results showed counts of *V. vulnificus* MO6-24, a clinical isolate, were 2 logs lower than those of the environmental strains 304 and A-9 after 9 h in the IC. Counts in the IC did not increase until low-pH gastric contents were emptied, which took 2 h (Fig. 2). The environmental strain A-9 caused diarrhea in rabbits without septicemia at 10^9 CFU/loop, while the clinical strain MO6-24 caused death at the same dose (41). Since the present model is based on an in vitro study, virulence factors such as acid tolerance or adaptation, intestinal colonization, and invasion were not evaluated. After passage through mice, the 50% lethal doses for oyster and environmental *V. vulnificus* isolates were reduced 100- and 1,000-fold, suggesting that strains passed through the GI tract may increase in potential pathogenicity (25). In the present study, the clinical isolate was less acid tolerant and grew less than the environmental isolate or the oyster isolate in the model. While the differences in acid tolerance and growth were not significant ($P < 0.05$), these observations suggest that the clinical strain has no selective advantage from human carriage or may have lost it during lengthy laboratory storage and numerous subcultures. The role of survival and/or growth during gastrointestinal transit in human infection should not be based on the performance of a single strain. Present results on acid toler-

ance may be less important in human infection than other virulence traits.

The pH values obtained in the present GI model compare well to in vivo results reported elsewhere (21, 32). The overall pH means of GC and IC in the present study were 2.7 and 6.8, which compare with the overall median fasting gastric pH of 1.7 in young and healthy men and women (11). During a meal, gastric pH increases to a median value of 5.0 (11). The overall fasting duodenal pH is 6.1, which increases to 6.3 during a meal (32).

In the present study, numbers of *V. vulnificus* organisms delivered from the GC increased continuously for 9 h in the IC (Fig. 2), presumably because bile does not greatly affect survival and growth of *V. vulnificus* (28). This finding suggests that viable *V. vulnificus* cells can be delivered into the small intestine if gastric emptying occurs soon after ingestion and that they will multiply rapidly in the intestine. Because all strains responded similarly in the gastric model, the risk of infection would appear to be proportional to dosage.

Gastric pH increased to 7.7 instantly after addition of antacid and oyster homogenate and remained above 5.5 for 2 h in the present study. The duration of the antacid effect may last longer than 2 h (15). *V. vulnificus* numbers in the IC containing antacid peaked sooner (4.5 to 6 h) than in the IC without antacid (9 h), because higher numbers of *V. vulnificus* organisms were delivered from the GC, and more rapid growth occurred probably due to the absence of a lag phase caused by acid stress (Fig. 3).

Behavior of *V. vulnificus* phage. Present results showing that *V. vulnificus* phage was more acid resistant in the GC than its host agrees with previous studies using acidified broth and SGF (27, 28). These findings suggest that *V. vulnificus* phage surviving the gastric barrier might subsequently affect populations of susceptible *V. vulnificus* in the GI tract as they enter log phase growth. The ecological role of coliphage lytic to *E. coli* in the human intestine has been studied (17). Fecal samples with low coliphage titers from healthy subjects contained mainly temperate phages, while those with high titers from patients under medical treatment contained mainly virulent phages. Survival time of phages lytic to enteropathogenic *E. coli* in pH 2.0 milk whey at 37°C ranged from 0.5 to 5 min, compared to 5 to 60 min for that of their host cells, suggesting that *E. coli* phages were less acid resistant than their host cells (40). While we could not find studies regarding survival of bacteriophage in the human GI tract containing antacid, Smith et al. (40) showed that adding $CaCO_3$ to milk whey inoculated with *E. coli* phages enhanced their survival as their numbers in the small intestines of calves 5 or 10 h after feeding were approximately four to five times higher than those without $CaCO_3$.

Conclusion. *V. vulnificus* numbers were reduced by 5 logs within 30 min in the GC, but surviving cells grew well in the IC, reaching 10^6 to 10^9 CFU/ml within 9 h. Differences in acid tolerance among the three strains were minor; in fact the one clinical strain did not perform as well as either of the two environmental strains with regard to acid tolerance in GC or growth in IC. This observation suggests that gastric emptying rate may be more important in *V. vulnificus* infections than differences in acid tolerance. Unpublished U.S. Food and Drug Administration data indicate that *V. vulnificus* levels in Gulf Coast oysters at the point of consumption often exceed 10^4/g (D. W. Cook, personal communication). Thus, a meal of a single oyster could contain more than 10^5 *V. vulnificus* organisms, indicating that any gastric emptying within 30 min of consumption would readily deliver viable cells to the intestine where they could multiply. The use of antacids and probably acid blockers would substantially increase the chances of gastric survival and probably influence subsequent infection. Since *V. vulnificus* phages were more acid tolerant than their hosts they would likely be introduced into the small intestine simultaneously with *V. vulnificus*. However, the ability of the phages used in this study to lyse *V. vulnificus* in the small intestine is questionable as their culturability is greatly reduced in the absence of seawater in the media used for their propagation, but other phages may be less dependent on seawater. Certainly, the intriguing possibility that phages indigenous to oysters may help protect against *V. vulnificus* infections cannot be discounted and may help explain the low attack rate (<0.0001) in individuals with preexisting liver disease that consume raw Gulf Coast oysters (24).

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